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Flavones-bound in benzodiazepine site on $GABA_A$ receptor: Concomitant anxiolytic-like and cognitive-enhancing effects produced by Isovitexin and 6-C-glycoside-Diosmetin

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ABSTRACT

Increasing evidence suggests that flavones can modulate memory and anxiety-like behaviour. However, these therapeutic effects are inconsistent and induce of adverse effects, which have been associated with interactions at the Benzodiazepine (BZ)-binding site. To improve our understanding of flavone effects on memory and anxiety, we employed a plus-maze discriminative avoidance task. Furthermore, we evaluated the potential of the compounds in modulating $GABA_A$ receptors via BZ-binding site using molecular modelling studies. Adult male Wistar rats were treated 30 min before training session with Vicenin-2 (0.1 and 0.25 mg/kg), Vitexin (0.1 and 0.25 mg/kg), Isovitexin (0.1 and 0.25 mg/kg) and 0.1 mg/kg 6-C-glycoside-Diosmetin, vehicle and a $GABA_A$ receptor agonist. The analysis of the time spent in the non-aversive vs aversive enclosed arms during the test session and percentage of time in the open arms within the training session revealed that treatment with Isovitexin and 6-C-glycoside-Diosmetin had memory-enhancing and anxiolytic-like effects ($P < 0.001$). In contrast, treatment with a higher dose of Diazepam impaired short-and long-term memory when it alleviated anxiety level. Docking studies revealed that flavones docked in a very similar way to that observed to the Diazepam, except by a lack of interaction in residue α1His101 in the BZ-binding site on $GABA_A$ receptors, which may be related to memory-enhancing effect. The occurrence of the α_1His101 interaction could justify the memory-impairing observed following Diazepam treatment. These findings provide the first evidence that Isovitexin and 6-C-glycoside-Diosmetin could exert their memory-enhancing and anxiolytic-like effects via $GABA_A$ receptor modulation, which likely occurs via their benzodiazepine-binding site.

1. Introduction

Anxiety disorders are the most common psychiatry disorders worldwide. Approximately one-third of the world population will suffer from at least one anxiety episode in their lifetime (WHO International Consortium in Psychiatric Epidemiology, 2000). Anxious individuals report impairments in executive functioning and episodic memory, as well as difficulty concentrating (Balderston et al., 2017; Vytal et al., 2012). Thus, understanding the interplay between anxiety and memory is important to establish appropriate therapeutic strategies for anxiety disorders.

Benzodiazepines (BZs) are the most common class of psychoactive drugs used to treat human anxiety (Griffin et al., 2013; Rudolph and Knoflach, 2011). BZs exert their actions via a modulatory binding site (the benzodiazepine binding site; BZ-binding site) that is present in different subtypes of $GABA_A$ receptors ($GABA_A$Rs). Diazepam, a non-selective agonist, increases the number or the opening frequency of ion channels in the presence of GABA, thus exerting anxiolytic, sedative,

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anticonvulsant, myorelaxant and cognitive-impairing effects (de Oliveira et al., 2014, 2015; Griffin et al., 2013; Rich et al., 2006; Verwey et al., 2005).

$GABA_A$ receptors belong to the superfamily of nicotinic-acetylcholine receptors (nAChRs) and are chloride-ion channels composed of one γ-, two β- and two α-subunits that may belong to different subunit classes. These subunits include α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, θ, and ρ_{1-3}, and the most abundant in the brain is the $\alpha_1\beta_2\gamma_2$ subtype $GABA_AR$ (Sieghart and Sperk, 2002; Stephenson, 1995). The BZ site is situated at the interface of α1, α2, α3, or α5 subunits and a γ subunit (Bergmann et al., 2013; Richter et al., 2012). Studies of functions involving punctual mutations of specific α subunits have provided invaluable information regarding the functions of different $GABA_A$ receptor subtypes. In these studies, the "missing" Diazepam actions in mice carrying the mutated α subunit are ascribed to the mutant α subunit. For example, in Diazepam α1 (H101R)-treated mice, the amnestic and sedative actions of diazepam were absent; however, its anxiolytic-like action was preserved (Rudolph et al., 1999; Rudolph and Mohler, 2004). There is substantial interest regarding the identification of new compounds with fewer side effects than classical benzodiazepines (for example, non-amnestic effects). In this search, an emerging area of interest is the activation of $GABA_A$ receptors by flavonoids (Goutman et al., 2003; Hanrahan et al., 2011).

Previous studies have identified a subclass of plant-derived compounds known as flavones, which exert actions on mammalian cognition (de Oliveira et al., 2014; Spencer, 2008, 2009; Wang et al., 2007, 2011) and anxiety disorders (Wang et al., 2007; Zhang et al., 2012). Vicenin-2 (apigenin-6,8-di-C-glycopyranoside), Vitexin (apigenin-8C-glucoside), Isovitexin (apigenin-6-C-glucoside) and 6-C-glycoside-Diosmetin are flavonoid derivatives isolated from the stem bark of *Erythrina falcata* L., which has been used as an herbal medicine. The memory effects of Vicenin-2, Vitexin, Isovitexin and 6-C-glycoside-Diosmetin on the central nervous system have been investigated by our group. It has been reported that acute Vitexin, Isovitexin and 6-C-glycoside-Diosmetin-treated rats enhance the acquisition of fear memory; however, this was not the case for Vicenin-2 (de Oliveira et al., 2014). These memory-enhancing effects may be related to the ability of the flavone to modulate GABAergic neurotransmission in the brain. Moreover, previous studies from different laboratories have shown that flavones, such as 6,20-Dihydroxyflavone, apigenin, baicalein and baicalin, exhibited contradictory effects on memory and anxiety, which impaired/improved or had no significant effects on memory in healthy or B-amyloid induced-impairment models; moreover, the anxiolytic and anxiogenic-like effects may be mediated by activation of the benzodiazepine binding (BZ-binding) site of $GABA_A$ receptors. These differential effects on memory and anxiety appear to be related to subunits that contain $GABA_A$ receptors and the differential affinities of these drugs. However, the basis of flavone interactions in the BZ-binding site remains unknown.

Accordingly, as inconsistent effects on memory and anxiety have been attributed to flavones and a potential interaction in the BZ-binding site on $GABA_A$ receptors, this study was designed to (i) concomitantly assess the effects of Vicenin-2, Vitexin, Isovitexin and 6-C-glycoside-Diosmetin treatment on memory, as well as anxiety-like and locomotor activity effects using the plus-maze discriminative avoidance task (PM-DAT), and (ii) evaluate the modulatory action of these flavones on the benzodiazepine binding site of $GABA_A$ receptors using molecular docking studies.

2. Materials and methods

2.1. Animals

A total of 80 male *Wistar* rats from 10 to 12 weeks old were obtained from the Center for the Development of Experimental Medicine and Biology (CEDEME, Federal University of Sao Paulo, SP, Brazil). All animals were housed 5 animals/cage, under controlled lighting with a 12 h light/dark cycle, temperature (21 °C ± 2 °C) and relative humidity (53% ± 2%). The experiments were performed in the light phase of the cycle. Animals were allowed access to food and water ad libitum; all procedures were conducted in accordance with the Brazilian law for the use of animals in scientific research (No. 11.794), as recommended by the guidelines set by the National Institutes of Health Guide for the Care and Use of Nonhuman Animals in Research. The protocol was approved by the local Committee Governing the Ethics on the use of Animal Experimentation of the Federal University of Sao Paulo (CEUA, approval no. 840560/2014).

2.2. Compounds

Valium® (Diazepam) was purchased from Roche (São Paulo, Brazil), and Tween® 80 was obtained from Synth (Diadema, Brazil). Vicenin-2, Vitexin, Isovitexin and 6-C-glycoside-Diosmetin were obtained from the Institute of Chemistry, Nuclei of Bioassay, Biosynthesis and Ecophysiology of Natural Products (NuBBE), Sao Paulo State University, UNESP, Araraquara, SP, Brazil (de Oliveira et al., 2014).

2.3. Experimental protocol and systemic administration

Rats were randomly assigned to 10 groups (n = 8/group) as follows: 12% Tween® 80 (negative control), Diazepam (4 mg/kg and 0.10 mg/kg, positive control), Vitexin (0.25 mg/kg and 0.10 mg/kg), Isovitexin (0.25 mg/kg and 0.10 mg/kg), Vicenin-2 (0.25 mg/kg and 0.10 mg/kg) and 6-C-glycoside-Diosmetin (0.10 mg/kg). The Diazepam and flavones were re-suspended in water that contained 12% Tween 80®. The dose ranges of all substances were selected according to previous studies conducted in our laboratory (de Oliveira et al., 2014). Flavones, Diazepam and 12% Tween® 80 were administered orally via an intragastric tube (IG) at 30 min before the training session (Tr).

2.4. Plus-maze discriminative avoidance task (PM-DAT)

2.4.1. Behavioural apparatus

The plus-maze discriminative avoidance task apparatus was composed of wood, which contained two enclosed arms (aversive enclosed arm and non-aversive enclosed arm (50 × 12 × 40 cm)) opposite to two open arms (50 × 12 cm). A lamp (100 W) and one speaker (that emitted 85 dB) were placed over one of the aversive enclosed arm (Fig. 1) (Frussa-Filho et al., 2016; Silva and Frussa-Filho, 2000).

2.4.2. Behavioural procedure

All rats were individually transferred and maintained in the adjacent room with a controlled intensity of light at 30 min before the Training session (Tr) and Test session (T). Behavioural sessions were recorded using a Panasonic® camera model SDR-T51 (Panasonic®, Sao Paulo, Brazil) fixed on the ceiling above the apparatus. In both sessions, the apparatus was cleaned with 10% ethanol solution prior to the introduction of a rat.

2.4.2.1. Training session (Tr). Rats were individually placed in the centre of the apparatus (facing the space between both open arms) and over a period of 10 min. When the rats entered with the four paws in the aversive enclosed arm (AEA), they received the aversive stimuli (a light; 100-Watt lamp and 85 dB sound, produced by a speaker) (Fig. 1A). The aversive stimuli were administered until the rats left the aversive enclosed arm. The percentage of time spent in the aversive enclosed arm was used as a measure of the acquisition of conditioned fear and short-memory. We simultaneously analysed the anxiety-like behaviour in rats by the percentage of time spent in the open arm (OA) and a time risk assessment. Therefore, short-term memory was calculated as a percentage of time spent in the aversive enclosed arm (% AEA) according the following equation: % AEA = [(AEA/(AEA +

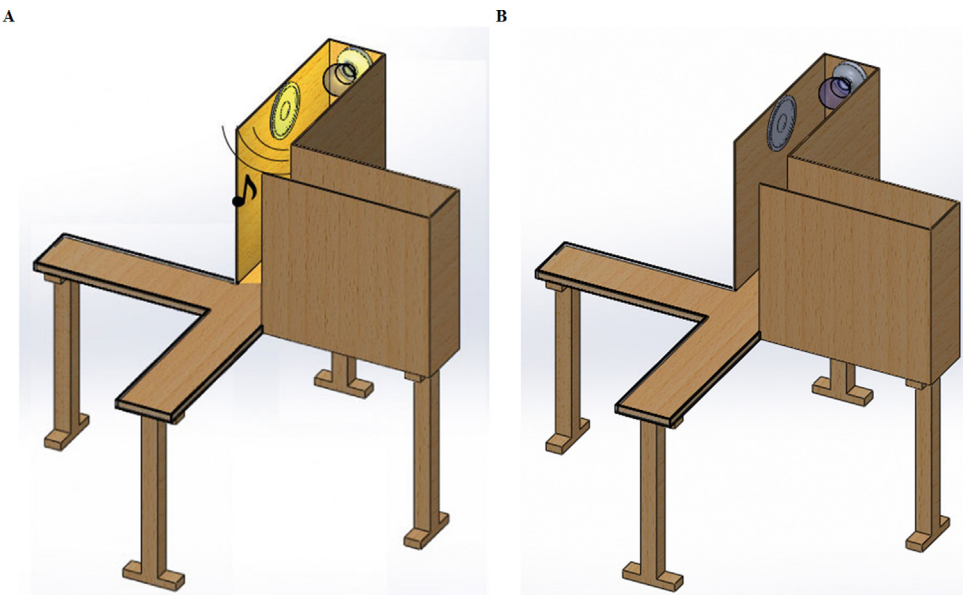

Fig. 1. Plus-maze discriminative avoidance task (PM-DAT). (A) Rats were subjected to one training session. Rats received the aversive stimuli (a light; 100 W lamp and 85 dB sound – (US), produced by a speaker associated with the context (CS)) when they entered with four paws in the aversive enclosed arm (AV). (B) After twenty-four hours, the rats were submitted to one test session. No light and sound were administered in the test session. Time spent in the AV, non-AV, OA, risk assessment and number entries were measured in both sessions. Open arms (50 cm × 12 cm); enclosed arms (50 cm × 12 cm × 40 cm). Plexiglas was used in the open arms. A video camera mounted above the maze was used to record both sessions.

NAEA)) × 100], where % AEA is the time spent in aversive enclosed arm/time spent in both enclosed arms [aversive enclosed arms (AEA) and non aversive enclosed arms (NAEA)]. Anxiety-like behaviours were calculated as percentage of the time spent in the open arms using the equation % OA = [(OA/(OA + AEA + NAEA) × 100)], where % OA correspond is the time spent in the open arms/time spent in both open and enclosed arms. Furthermore, we consider a time performing risk assessment, which consists of stretch-attend postures and behavioural assessment, has been used to determine when rodents evaluate/avoid the open arms (Carobrez and Bertoglio, 2005). The total number of entries into any of the arms (an entry was defined as the entry of 4 paws into an arm) was used to evaluate the motor activity.

2.4.2.2. Test session (T). The test was performed 24 h after the training session, and the time spent in the aversive enclosed arm vs non aversive enclosed arm, over a period of 3 min, was used as a measure of the retention of long-term memory (LTM), according described by Frussa-Filho and collaborators (Frussa-Filho et al., 2016; Silva and Frussa-Filho, 2000). During the test session, no aversive stimuli were administered. The total number of entries into any of the arms was evaluated again.

2.4.3. Data analysis

All training and test session data were presented as the mean ± standard error of the mean (S.E.M.), and the data were validated using the normality test (Shapiro-Wilk test). Comparisons among the percentage of time spent (minute to minute) in the aversive enclosed arm and discrimination between the aversive and non-aversive enclosed arm were analysed by two-way repeated measure analysis of variance (two-way RM ANOVA) followed by Bonferroni's post hoc test. The percent time spent in the open arms, percent of the whole time spent in the aversive enclosed arm, the number of entries and the number risk assessment were compared via one-way ANOVA followed by Bonferroni's post hoc test. All statistical analyses were performed using the GraphPad Prism statistical program (Graph Pad 6.0 Software®, San Diego, CA, USA). Differences at the $P < 0.05$ level were considered statistically significant.

2.5. Molecular docking studies

Molecular docking was used to predict the interaction between the flavones and the $GABA_A$ receptor via their benzodiazepine-binding (BZ-binding) site, which would allow us to understand the behaviour of the flavones in the BZ-binding site, establish which interactions may be involved in this recognition process and elucidate the biological processes, in particular, memory and anxiety.

Three-dimensional structures of Vicenin-2 (**2**), Vitexin (**3**), Isovitexin (**4**) and 6-C-glycoside-Diosmetin (**5**) (Fig. 2D) were constructed using the GaussView 5.0 Sketcher Module (GaussView 5.0, Gaussian Inc., Shawnee Mission, KS, USA) and were subsequently optimized using the MM+ force field and semi-empirical method AM1 implemented in the same software. GOLD 5.4.1 Software® (GOLD Suite Intuitive Protein-Ligand Docking Package, Cambridge, UK) was used to perform the rigid docking analyses and employed a standard protocol obtained from previous re-docking studies, regarding Diazepam as the original ligand. The $GABA_A$ receptor proteins were pre-processed by adding missing Hydrogens and removing all water molecules from the crystal structure (PDB ID: 3RIF). A centroid point was established among the residues Ser204 and Thr206 from chain α1 (C-loop – (defined as an H2 and H1' interaction, respectively)) and Thr142 from chain γ2 (D-loop – defined as an H1 interaction)) as these residues have been reported as a fundamental part of the benzodiazepine-binding pocket of Diazepam (**1**) in the $GABA_A$ receptor model, Fig. 2A–C (Bergmann et al., 2013; Richter et al., 2012). All residues located at 10 Å from the centroid were considered in the docking analysis. The Tyr159, Try209 and Phe99 residues from chain α1 (defined as an L1 pocket), Phe77 from chain γ2 (defined as an L3 pocket) and the positively charged His101 from chain α1 (defined as an L3 pocket) (Fig. 2A) were considered in this context as important points of hydrophobic and electrostatic interactions (Bergmann et al., 2013; Richter et al., 2012). Vicenin-2 (**2**), Vitexin (**3**), Isovitexin (**4**) and 6-C-glycoside-Diosmetin (**5**) (Fig. 2D) were docked into the $GABA_A$ receptor model, thus generating a set of 100 poses, which were scored and ranked regarding two different score functions implemented in Gold 5.4.1: GoldScore and ChemScore. The top ten poses from the ligand-receptor complex were ranked considering the alignment/orientation in the active site regarding the potential intermolecular interactions (distance values) combined with information from the GoldScore and ChemScore fitness rank position. The cut off value was 4.0 Å. Visualization of the best ligand-receptor complex modes was performed using Maestro 9.4 Software® (Schrödinger, Inc., LLC, Academic License).

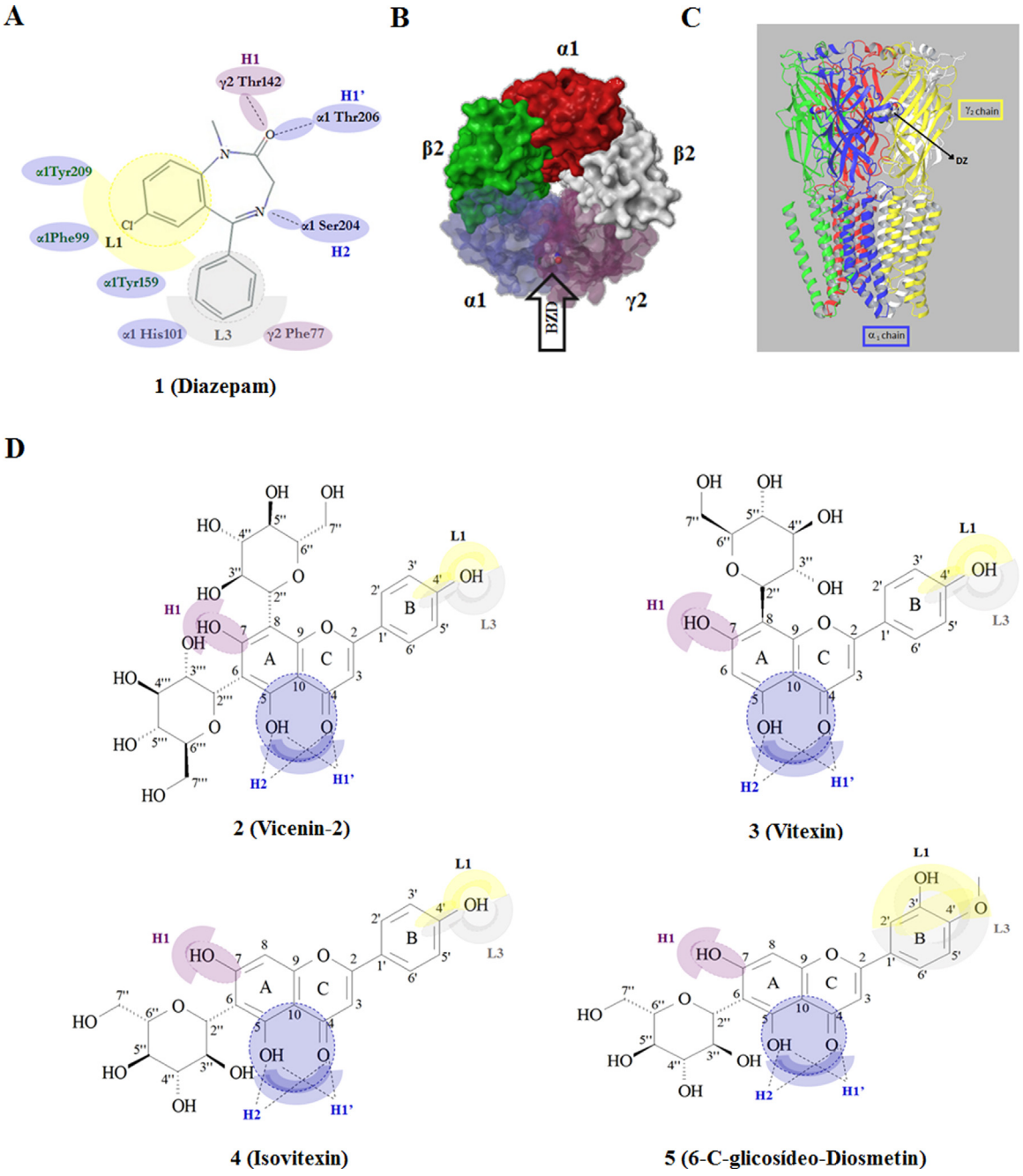

Fig. 2. Chemical and molecular structures of Diazepam-bound $GABA_AR$ and Flavones. (A) The pharmacophoric features L1, L3, H1 and H2 superimposed on the structure of diazepam (1). (B) Top view shows the pentameric assembly of α1, β2 and γ2 subunits and the benzodiazepine-binding site (BZD). (C) Side view illustrates the extracellular domain (ECD) where BZD binds and the transmembrane domain (TMD). (D) Molecular structures of flavones used in this study and the pharmacophoric features L1, L3, H1 and H2.

3. Results

3.1. Effects of flavones in short-term and long-term memory

The effects of Vicenin-2, Vitexin, Isovitexin-treated (0.25 mg/kg and 0.10 mg/kg), 0.10 mg/kg 6-C-glycoside-Diosmetin and the control treatment (12% Tween® 80, 4 mg/kg Diazepam and 0.10 mg/kg Diazepam) in the training session (short-term memory) and test session (long-term memory) are shown in Fig. 3.

In the training session, one-way ANOVA followed by Bonferroni's test considering the % time spent in the aversive enclosed arm indicated a significant difference among the groups [$F_{(3.844,26.91)} = 39.35$, $P < 0.0001$] (Fig. 3A). The rats treated with 4.0 mg/kg Diazepam exhibited a significant increase in the percentage time total in the aversive enclosed arm (33.45 ± 1.20, $P < 0.0001$) compared to the rats treated with Tween® 80 (15.53 ± 0.69), 0.10 mg/kg Diazepam (15.37 ± 0.74), 0.10 mg/kg Vicenin-2 (12.23 ± 1.61), 0.25 mg/kg Vicenin-2 (15.44 ± 0.74), 0.10 mg/kg Vitexin (10.91 ± 1.57), 0.25 mg/kg Vitexin (9.176 ± 1.52), 0.10 mg/kg Isovitexin (17.31 ± 0.78), 0.25 mg/kg Isovitexin (18.53 ± 1.22) and 0.10 mg/kg 6-C-glycoside-Diosmetin (19.58 ± 1.01). Fig. 3B presents the analysis of the percentage of time in the aversive enclosed arm (minute to minute). Two-way RM ANOVA indicated a significant interaction between groups and time factors [$F_{(81,567)} = 1.551$, $P = 0.0026$], an effect of groups [$F_{(9,63)} = 56.81$, $P < 0.0001$] and an effect of time [$F_{(9,63)} = 28.53$, $P < 0.0001$]. A comparison within the training session

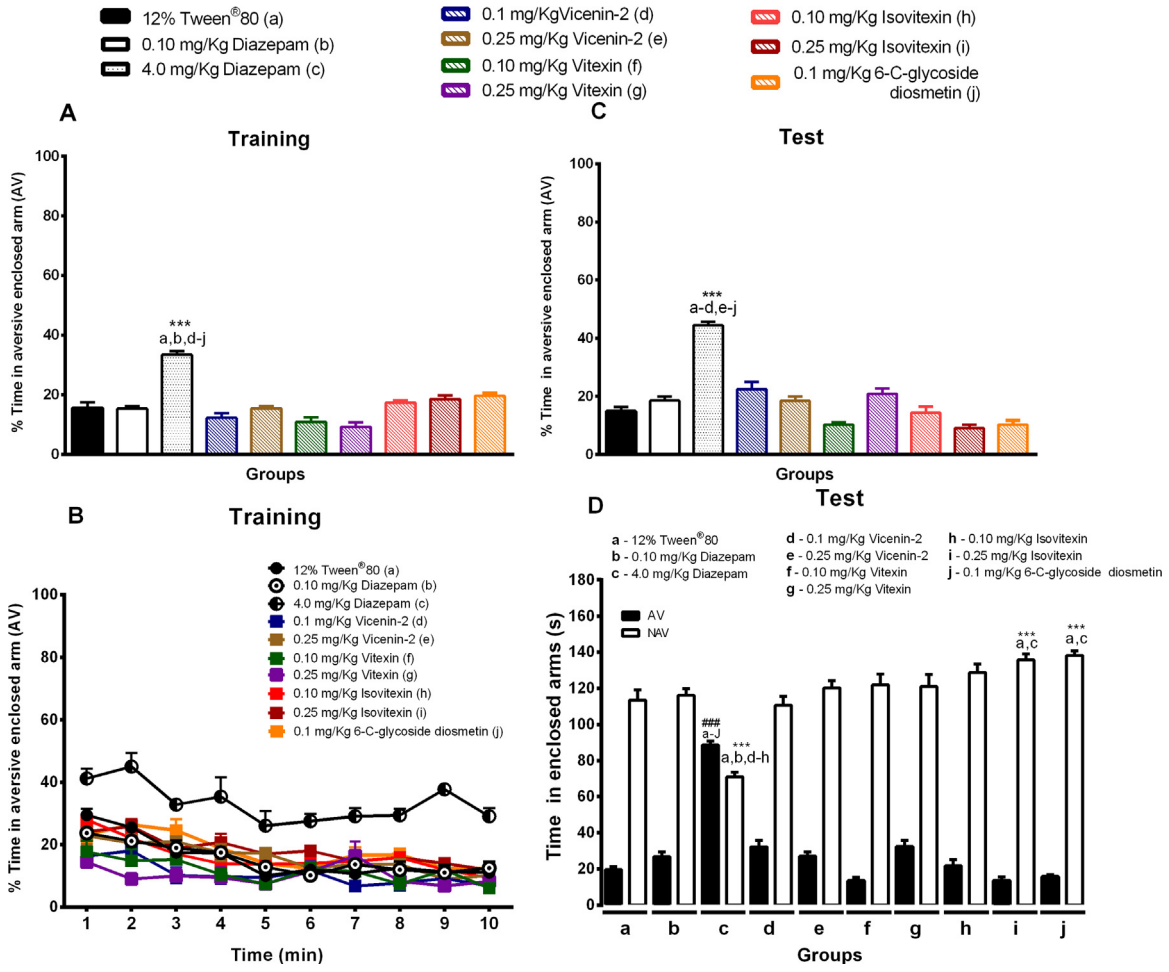

Fig. 3. Effects of acute treatment with Vicenin-2, Vitexin, Isovitexin and 6-C-glycoside-Diosmetin, as well as the negative (Tween® 80) and positive control (Diazepam), on short-term and long-term memory evaluated by the plus-maze discriminative avoidance task (PM-DAT). (A) Percentage of time in the aversive enclosed arm in the training session (top left panel); (B) Percentage of time in the aversive enclosed arm (minute to minute) in the training session (bottom left panel); (C) Percentage of time in the aversive enclosed arm in the test session (top right panel); (D) Time in enclosed arms in the test (both aversive (AEA) and non-aversive NAEA) session (bottom right panel). The values for PM-DAT as the mean values (± S.E.M.). The flavones, Diazepam and Tween® 80 were administered 30 min prior to the training sessions. No drugs were administered during the test. Differences among groups were represented by *** $P < 0.0001$, according to ANOVA followed by post hoc Bonferroni's test (A, C) and intra-group comparison (B), using GraphPad Prism Software®. Comparison between groups in the NAEA ($^{###}P < 0.0001$) and AEA (*** $P < 0.0001$), according to Two-way ANOVA followed by *post hoc* Bonferroni's test (D). Different lower case above the bars indicate experimental groups (a) 12% Tween 80, (b) 0.1 mg/kg diazepam, (c) 4 mg/kg diazepam (d) 0.1 mg/kg vicenin-2, (e) 0.25 mg/kg vicenin-2, (f) 0.10 mg/kg vitexin, (g) 0.25 mg/kg vitexin, (h) 0.10 mg/kg isovitexin, (i) 0.25 mg/kg isovitexin, (j) 0.10 mg/kg 6-C-glycoside-Diosmetin.

indicated that all groups decreased over time the % aversive enclosed arm ($P < 0.0001$), with the exception of the 0.25 Vitexin group (Fig. 3B). Moreover, all groups spent less time in the aversive enclosed arm than the non-aversive enclosed arm (Supplementary Fig. 1A). The groups treated with 4.0 mg/kg Diazepam, 0.10 and 0.25 mg/kg Isovitexin and 0.10C-glycoside Diosmetin spent a greater percentage of time in the open arm, as shown in Supplementary Fig. 1A. Overall, our data show Diazepam treatment at the dose 4.0 mg/kg impaired short-term memory in the PM-DAT task, as the total time spent in the aversive enclosed arm was higher than that in the Tween group. Furthermore, the rats treated with 0.25 mg/kg Vitexin were not able to discriminate the aversive enclosed arm and non aversive enclosed arm because the time spent in the aversive enclosed arm was similar during the training session.

Fig. 3C shows the % time spent in the aversive enclosed arm in the test session. One-way ANOVA followed by Bonferroni's test indicated significant difference among the groups [$F_{(3.328,23.30)} = 42.52$, ($P < 0.0001$)]. The rats that received 4.0 mg/kg Diazepam showed an increased % aversive enclosed arm total (44.44 ± 1.24, $P < 0.0001$) compared to Tween (14.87 ± 1.55), 0.10 mg/kg Diazepam (18.56 ± 1.37), 0.10 mg/kg Vicenin-2 (22.51 ± 2.45), 0.25 mg/kg Vicenin-2 (18.45 ± 1.52), 0.1 mg/kg Vitexin (10.20 ± 0.86), 0.25 mg/kg Vitexin (20.88 ± 1.80), 0.10 mg/kg Isovitexin (14.36 ± 2.07), 0.25 mg/kg Isovitexin (9.02 ± 1.27) and 0.10 mg/kg 6-C-glycoside-Diosmetin (10.23 ± 1.55), which suggested that 4.0 mg/kg Diazepam impairs retention long-term memory. Corroborating this data, two-way RM ANOVA to discriminate between both enclosed arms (aversive vs non-aversive, Fig. 3D) showed a significant arm type × group interaction [$F_{(9,70)} = 29.70$, $P < 0.0001$], an effect of groups [$F_{(9,70)} = 3.397$, $P = 0.0016$] and effect of arm type [$F_{(9,70)} = 2668$, $P < 0.0001$]. Bonferroni's post hoc test indicated that all groups spent significantly less time in the aversive enclosed arm than in the non-aversive enclosed arm during the test ($P < 0.0001$), which indicates memory retrieval, with the exception of the 4.0 mg/kg Diazepam group ($P > 0.05$). Analyses of the time spent in the non-aversive enclosed arm for each group indicated that the rats treated with 0.25 mg/kg Isovitexin (135.8 ± 3.29, $P < 0.0001$) and 0.10 mg/kg 6-C-glycoside-Diosmetin (138.1 ± 2.56, $P < 0.0001$) exhibited an increase in the spent time compared to the Tween (113.5 ± 5.56), 0.10 mg/kg Diazepam (116.1 ± 3.65) and 4.0 mg/kg Diazepam (88.63 ± 2.04).

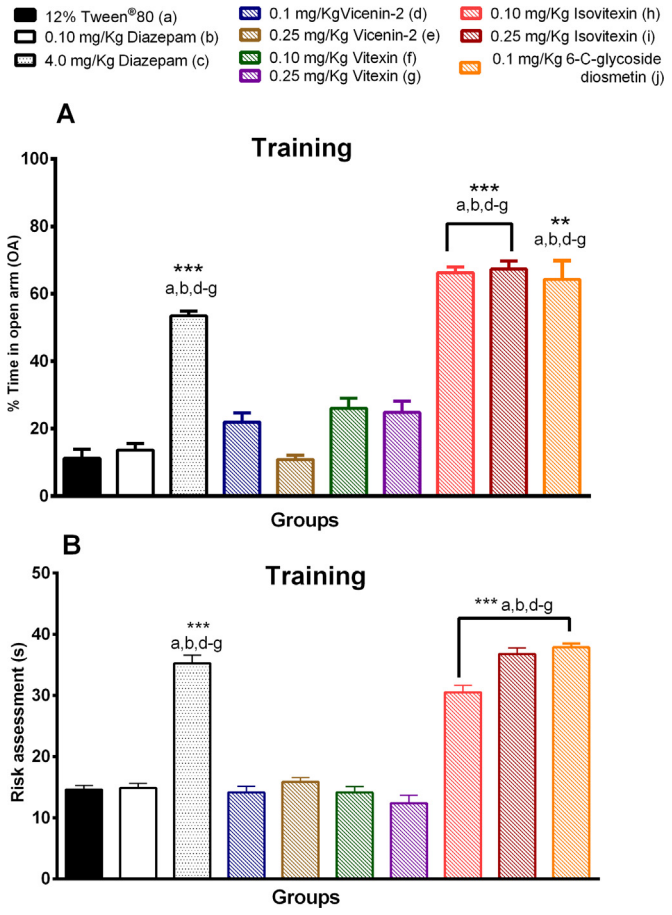

Fig. 4. Effects of acute treatment with Vicenin-2, Vitexin, Isovitexin and 6-C-glycoside-Diosmetin, as well as the negative (Tween® 80) and positive control (Diazepam), on anxiety-like behaviour evaluated via the plus-maze discriminative avoidance task (PM-DAT). (A) Percentage of time spent in the open arms (OA) in the training session and (B) Risk assessment (time in seconds) in the training session. The values for the PM-DAT as the mean values (± S.E.M.). Differences among groups were represented by ** $P <$ 0.001 and *** $P <$ 0.0001, according to ANOVA followed by post hoc Bonferroni's test using GraphPad Prism Software®. The flavones, Diazepam and Tween® 80 were administered 30 min prior to the training sessions. Different lower case letters above the bars indicate experimental groups (a) 12% Tween 80, (b) 0.1 mg/kg diazepam, (c) 4 mg/kg diazepam (d) 0.1 mg/kg vicenin-2, (e) 0.25 mg/kg vicenin-2, (f) 0.10 mg/kg vitexin, (g) 0.25 mg/kg vitexin, (h) 0.10 mg/kg isovitexin, (i) 0.25 mg/kg isovitexin, (j) 0.10 mg/kg 6-C-glycoside-Diosmetin.

However, the rats treated with 4.0 mg/kg Diazepam exhibited a reduced time spent in the non-aversive enclosed arm compared to the Tween group ($P <$ 0.0001), 0.10 mg/kg Diazepam ($P <$ 0.0001) and all flavone-treated rats ($P <$ 0.0001). Furthermore, the 4.0 mg/kg Diazepam-treated group exhibited a greater spent time in the aversive enclosed arm than all groups (Supplementary Fig. 1B). In summary, treatment with 0.25 mg/kg Isovitexin and 0.10 mg/kg 6-C-glycoside-Diosmetin enhanced the retention of long-term memory. In contrast, the group treated with 4.0 mg/kg Diazepam exhibited an impaired retention of memory.

3.2. Effects of flavones on anxiety-like behaviour

Simultaneously with the memory analysis, we analysed the time risk assessment and time spent in the open arm, which represents anxiety-like behaviours, during the training session for all control and flavones (Fig. 4). In the training session, one-way ANOVA followed by Bonferroni's test revealed a significant difference among the groups regarding the % OA [$F_{(2.629,18.41)} = 74.35$, $P <$ 0.0001] (Fig. 4A). The rats treated with 4.0 mg/kg Diazepam (52.83 ± 1.40, $P <$ 0.0001), 0.10 mg/kg Isovitexin (65.50 ± 1.70, $P <$ 0.0001), 0.25 mg/kg Isovitexin (66.67 ± 2.47, $P <$ 0.0001) and 0.1 mg/kg 6-C-glycoside-Diosmetin (70.75 ± 5.60, $P <$ 0.001) exhibited a significant increase in the % open arm compared to the Tween (10.67 ± 0.90), 0.10 mg/kg Diazepam (13.92 ± 0.69), 0.1 mg/kg Vicenin-2 (21.34 ± 2.71), 0.25 mg/kg Vicenin-2 (10.92 ± 1.24), 0.10 mg/kg Vitexin (25.00 ± 2.99) and 0.25 mg/kg Vitexin (24.83 ± 3.37) groups. No differences were identified between the groups treated with 0.10 mg/kg Diazepam, Vicenin-2 (0.10 or 0.25 mg/kg), Vitexin (0.10 or 0.25 mg/kg) and Tween ($P >$ 0.05). Corroborating this data, the One-way ANOVA regarding risk assessment (time in seconds) indicated a significant difference among the groups [$F_{(3.542,24.80)} = 114.9$, $P <$ 0.0001] (Fig. 4B). Bonferroni's post hoc test indicated that the rats treated with 4.0 mg/kg Diazepam (35.25 ± 1.33, $P <$ 0.0001), 0.10 mg/kg Isovitexin (30.50 ± 1.15, $P <$ 0.0001), 0.25 mg/kg Isovitexin (36.75 ± 1.01, $P <$ 0.0001) and 0.10 mg/kg 6-C-glycoside-Diosmetin (37.88 ± 0.63, $P <$ 0.001) exhibited a significant increase in the time performing risk assessment compared to the rats treated with Tween (14.63 ± 0.67), 0.10 mg/kg Diazepam (14.88 ± 0.76), 0.10 mg/kg Vicenin-2 (14.13 ± 1.02), 0.25 mg/kg Vicenin-2 (15.88 ± 0.66), 0.10 mg/kg Vitexin (14.13 ± 0.97) and 0.25 mg/kg Vitexin (12.38 ± 1.28). No differences were identified in the groups treated with 0.10 mg/kg Diazepam and Vicenin-2 (0.10 or 0.25 mg/kg) compared with Tween ($P >$ 0.05). In summary, these data indicated an anxiolytic-like behaviour effect for 4.0 mg/kg Diazepam, Isovitexin (at both doses) and 0.10 mg/kg 6-C-glycoside-Diosmetin.

3.3. Effects on locomotion

The motor activities of the control-groups and treated-groups are shown in Table 1. No differences were found in the total number of entries in the enclosed arms or open arms during the training session [$F_{(9,80)} = 1.302$, ($P =$ 0.2493)]. However, rats treated with 4 mg/kg Diazepam showed increased total number of entries within test session when compared to 0.10 mg/kg Vicenin-2 and 0.10 mg/kg Vitexin groups [$F_{(9,80)} = 3.712$, ($P =$ 0.0006)].

3.4. Molecular docking studies to predict a binding mode for flavones in $GABA_A$ receptor via their benzodiazepine-binding site

The molecular docking procedure was applied considering that all flavones (**2–5,** Fig. 2D) of this study are agonists of $GABA_A$ receptor, as previously reported for other flavones (Dekermendjian et al., 1999; Marder et al., 2001). As described, flavones were docked to the ligand BZ-binding site following the model reported for Diazepam (Bergmann et al., 2013; Richter et al., 2012), without constraints and offering, as reference to the dock, a centroid point obtained regarding the Ser204

Table 1
Total number of entries in any of the arms in training and test session.

Groups	Sessions	
	Training	Test
12% Tween® 80	6.44 ± 0.5031	4.60 ± 0.2583
0.10 mg/kg Diazepam	6.00 ± 0.7071	4.54 ± 0.2393
4.0 mg/kg Diazepam	5.66 ± 0.5000	5.38 ± 0.4331
0.10 mg/kg Vicenin-2	5.66 ± 0.5270	2.90 ± 0.5907
0.25 mg/kg Vicenin-2	7.44 ± 0.4444	4.61 ± 0.2321
0.10 mg/kg Vitexin	5.66 ± 0.5270	3.11 ± 0.5636
0.25 mg/kg Vitexin	6.88 ± 0.5122	4.61 ± 0.2321
0.10 mg/kg Isovitexin	6.88 ± 0.5879	4.53 ± 0.2376
0.25 mg/kg Isovitexin	7.0 ± 0.4714	4.82 ± 0.6129
0.10 mg/kg 6-C-glycoside-Diosmetin	7.55 ± 0.8012	4.12 ± 0.2602

The values for PM-DAT as the mean values (± S.E.M.).

and Thr206 residues from α1 chain and Thr142 from γ2 chain (Fig. 2A). The results were analysed to identify potential interactions such as those observed by Richter et al. (2012) and Bergmann et al. (2013). The hydrophobic residues α_1Tyr159, α_1Phe99, and γ_2Phe77 and the positively charged α_1His101 were reported by Richter et al. (2012) and Bergmann et al. (2013) as important points of interaction (Fig. 2A).

Flavones were considered potential ligands for the benzodiazepine binding site, which exhibited coincident poses with more than 60% frequency. We considered the best pose as the one that fulfilled the following criteria: the presence of ring B pointing towards the hydrophobic region of α_1Tyr159, α_1Phe99, and γ_2Phe77 and the positively charged α_1His101, best suitable energy scoring functions (Goldscore and Chemscore functions) and the following smaller distances in Å: (i) oxygen atom of the carbonyl group at ring C of the flavone and oxygen atoms of the α_1Thr206 and α_1Ser204 from the active binding site; (ii) oxygen atom of the hydroxyl group in ring A from the flavones and oxygen atoms of the same α_1Thr206 and α_1Ser204; and (iii) oxygen atom of the hydroxyl group in ring A of the flavone and oxygen atom of Thr142 in γ_2 chain of the active site (Fig. 2). Supplementary Tables 1–4 show these measured distances in Ângströms (Å) and the suitable energy scoring functions (GoldScore and ChemScore) for all investigated flavones (2–5).

Analysis of the theoretical binding mode of Vicenin-2 (**2**) showed the worst profile of interactions with the residues in the binding pocket, which resulted in a similar and predominant coincident pose in only 13% of the docked poses. Regarding these findings, Vicenin-2 was not considered a probable ligand of the $GABA_A$ receptor (Supplementary Fig. 2 and Supplementary Table 1). Similarly, Vitexin (3) does not show a good adjustment at the binding pocket, and the frequency of a coincident pose was of only 33% (Supplementary Fig. 2 and Supplementary Table 2).

In contrast, Isovitexin (**4**) was the best flavone adjusted to the BDZ binding site, and the frequency of a coincident pose was 73% (Fig. 5E and H), which was higher than the other flavones previously discussed. It showed a more similar theoretical binding mode to the BDZ. Strong interactions between the carbonyl oxygen at C_4 of ring C and the residues α_1Thr206 and α_1Ser204 (2.40 Å and 1.51 Å, respectively) were observed. The hydroxyl oxygen at C_5 of ring A also interacts with α_1Thr206 and α_1Ser204 (1.41 Å and 2.31 Å, respectively). Interestingly, these four described interactions form a quadrilateral geometry (Fig. 5B; Supplementary Table 3), which may reflect a strong affinity of this flavone for the $GABA_A$ receptor. The hydroxyl oxygen at C_7 of ring A approaches the γ_2Thr142 (Fig. 5B), which most mimics the BZ binding mode and interacts with γ_2Thr142. Ring B appears to be involved with γ_2Phe77 and α_1Phe99 of the hydrophobic pocket by means of aπ-π stacking interactions. The glycoside moiety at C_6 also showed different interactions with the binding site, most of them between the hydroxyl groups of the sugar and the residues γ_2Asp56 and γ_2Glu189 (1.38 Å). In contrast to the results obtained with Diazepam, the distance between the hydroxyl group from ring B and α_1His101 was larger than acceptable as a potential interaction (4.73 Å) (Fig. 5B).

6-C-glycoside-Diosmetin (**5**) also shows interactions between the carbonyl oxygen at C_4, ring C, and the α_1Thr206 and α_1Ser204 (2.94 Å and 1.07 Å, respectively), as well as between the hydroxyl oxygen at C_5, ring A, and α_1Thr206 and α_1Ser204 (2.04 Å and 2.11 Å, respectively). It is also possible to visualize the quadrilateral geometry of the interactions with 6-C-glycoside-Diosmetin (Fig. 5C; Supplementary Table 4), which may imply, as in the case of isovitexin, a better affinity to the BDZ binding site. In contrast to the other flavones, 6-C-glycoside-Diosmetin interacts with a stronger affinity with the hydrophobic pocket with its ring B groups. It is involved in a π-π stacking interaction with the residues γ_2Phe77 and α_1Phe99, as well as an H-bound interaction with the hydroxyl group of α_1Tyr159 (2.24 Å). In ring A, both the hydroxyl group at C_7 and the glycoside at C_6 strongly interact with

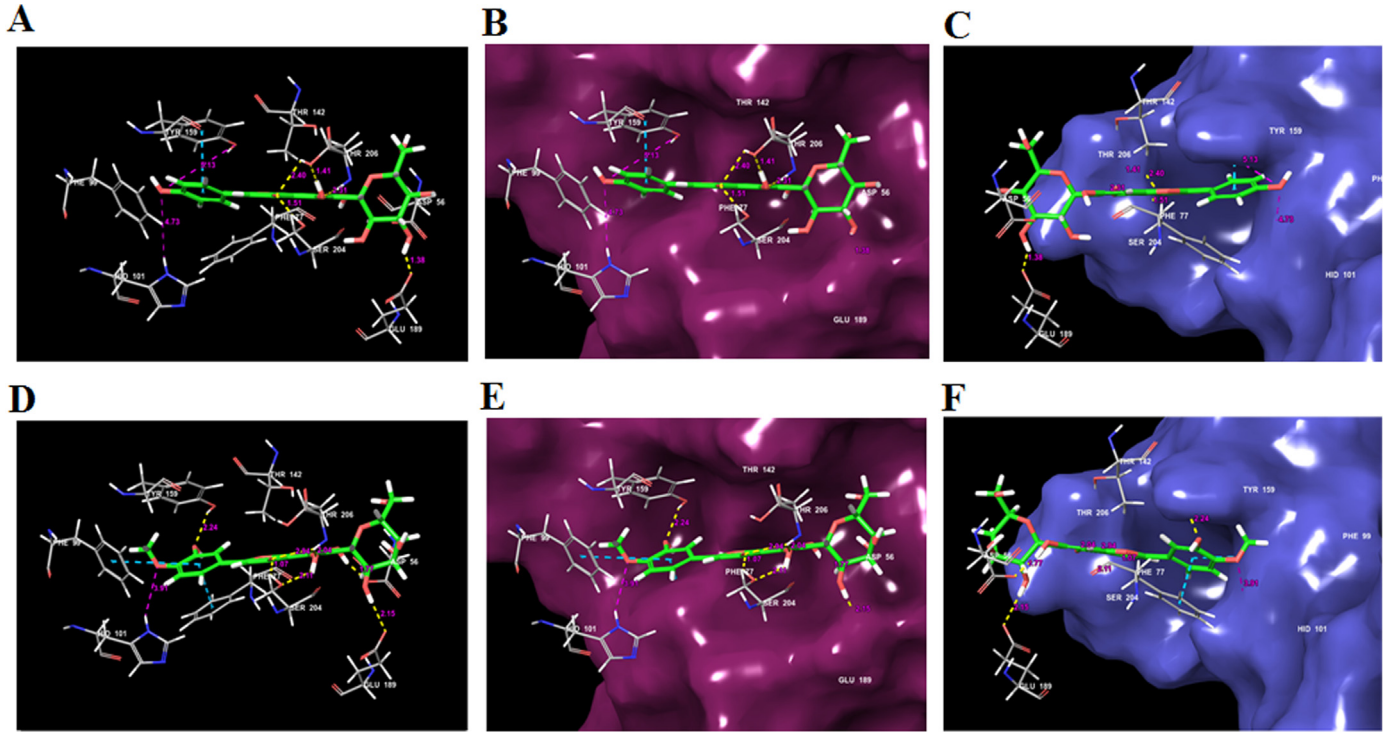

Fig. 5. Molecular docking of Isovitexin and 6-C-glycoside-Diosmetin in $GABA_AR$ via their benzodiazepine-biding site (BZD). Two-dimensional scheme shows Isovitexin (A) and 6-C-glycoside-Diosmetin (D) in BZD site. Anterior view of the Isovitexin (B) and 6-C-glycoside-Diosmetin (E) in molecular surface representation in the γ_2-subunit of the BZD site. Posterior view of Isovitexin (C) and 6-C-glycoside-Diosmetin (F) in molecular surface representation in the α_1-subunit of the BZD site. Representation shows the γ_2-subunit (maroon) and α_2-subunit (blue). Hydrogen-bond interactions are indicated as yellow dashed lines. Distances between two groups not interacting with each other are indicated as blue dashed lines or maroon dashed lines. Colour codes: green (ligand) or grey ($GABA_AR$ for carbon; blue for nitrogen; red for oxygen and white for hydrogen).

the carboxylic acid of γ_2Asp56 (1.77 Å). This flavone also does not present an interaction with α_1His101, being the closest distance between the oxygen from the methoxy group and this residue of 3.91 Å. This compound shows a very good adjustment to the binding site, and the frequency of a coincident pose was 60% (Fig. 5F and I).

4. Discussion

Our findings obtained using PM-DAT and molecular docking studies provide direct evidence of the modulatory effects of C-glycosylated flavones on memory and anxiety in an integrative manner. Three main findings emerge from this study. First, acute treatments with 0.25 mg/kg Isovitexin and 0.10 mg/kg 6-C-glycoside-Diosmetin exhibited enhanced long-term memory in relation to control animals, as indicated in the test session (evaluated by the time spent in the aversive enclosed arm compared to the time in the non-aversive enclosed arm). Second, rats treated with Isovitexin (at both doses) and 6-C-glycoside-Diosmetin showed concurrently anxiolytic-like behaviour effects. In contrast, 4 mg/kg Diazepam treatment impaired short-term and long-term memory when it alleviated anxiety levels. Third, the molecular docking results reveal, for the first time, an unexpected lack of interaction in residue α_1His101 in the benzodiazepine-binding site for Isovitexin and 6-C-glycoside-Diosmetin.

4.1. Flavone treatment prior to training session simultaneously modulates short- and long-term memory and anxiety-like behaviour

Flavones have been speculated to act as mnemonic drugs (de Oliveira et al., 2014); however, the anxiolytic properties of these flavones have not been investigated. Our data showed that the rats that were treated with Isovitexin (at both doses) and 6-C-glycoside-Diosmetin exhibited an important anxiolytic-like effect without preventing short and long-term memory in the PM-DAT task as observed for Diazepam. In the final minutes of the training session, they spent a short time exploring the aversive enclosed arm where the aversive stimulus is presented. Furthermore, in the retention test session we observed retention of LTM since Isovitexin- (at both doses) and 6-C-glycoside-Diosmetin–treaded rats spent significantly more time in the enclosed non-aversive arm when compared to enclosed aversive arms. The evidence of dual effects of the Isovitexin and with 6-C-glycoside-Diosmetin on anxiety and fear memory focused our attention on the therapeutic relevance of these data because these pharmacological agents might serve as an adjunct to the treatment of memory decline or anxiety disorders. Interestingly, the anxiolytic and memory effects were not necessarily modulated at the same site in the benzodiazepine-biding site (BZD). Thus, Isovitexin and 6-C-glycoside-Diosmetin enhanced memory levels, which may be related to optimal levels of anxiety required to obtain an adequate performance on the PM-DAT task, as previously described (Calzavara et al., 2004; Silva and Frussa-Filho, 2000). Our present data corroborate our previous finding that treatment with Isovitexin and 6-C-glycoside-Diosmetin improved retention test performance in a one-trial step-down inhibitory avoidance task (de Oliveira et al., 2014). Other studies have demonstrated beneficial effects of flavones in reducing anxiety levels in the Vogel task (Liao et al., 2003) and improving memory in a fear conditioning task (Wang et al., 2011). In this context, our current data are consistent with previous findings. Furthermore, the findings suggest a molecular mechanism for the anti-anxiety like behaviour and cognitive enhancing effect of Isovitexin and 6-C-glycoside-Diosmetin, as considered below.

Considering the efficacy of Diazepam in reducing anxiety symptoms and side effects commonly present, such as psychomotor, cognitive impairment and sedative effects, we employed Diazepam as a positive control in our study. The pharmacological properties of Diazepam on $GABA_A$ receptors appear to be mediated by the α1-subunit, which is involved in sedative and cognitive impairments (amnestic effect) (Bergmann et al., 2013; Rudolph and Mohler, 2004; Savic et al., 2009). Our results confirm that treatment with 4 mg/kg Diazepam had an anxiolytic-like effect and impaired short- and long-term memory retention evaluated in the training and test session, respectively, in the PM-DAT task and is in accordance with data described by Silva and colleagues (Silva et al., 2016). This effect is well-established in rodents studies in different tasks (de Oliveira et al., 2014, 2015; Silva et al., 2016) and human studies (Rich et al., 2006; Roy-Byrne et al., 1987; Verwey et al., 2005). Moreover, the increase in the total number of entries into any of the arms during testing suggest that the rats treated with Diazepam at the higher dose could not distinguish the aversive arms from the non-aversive arm in the test session in relation to rats treated with flavones (Vicenin-2 and Vitexin), which was interpreted as impaired memory retention, since no drugs were administered before the test session.

No anti-anxiety and memory-enhancing effects were identified for rats treated with Vicenin-2 and Vitexin. Although the risk assessment parameter is considered a sensitive measure of anxiety-like behaviour (Carobrez and Bertoglio, 2005), our results did not show an effect of treatment on this parameter and locomotor activity.

We suggest that three different possibilities may explain the concomitant anxiolytic-like and cognitive-enhancing effects produced by Isovitexin and 6-C-glycoside-Diosmetin. First, Isovitexin and 6-C-glycoside-Diosmetin may have produced anxiolytic-like effects via their own agonist in the α1 subunit on $GABA_A$ receptors, such as Diazepam. Second, the memory-enhancing effects produced by flavone may have been a consequence of a sensitivity in different types of α subunit-containing $GABA_A$ receptors. However, this hypothesis requires further corroboration through additional experiments. Third, another explanation for the memory-enhancing effects could be related to the lack of interaction in some residues from the α1 subunit on $GABA_A$ receptors. To investigate the first and third hypotheses, we evaluated the behaviour of C-glycosylated flavones on $GABA_A$ receptors via their BZ-binding site using a molecular modelling study.

4.2. Molecular docking studies

Vicenin-2, Vitexin, Isovitexin and 6-C-glycoside-Diosmetin were docked into the Benzodiazepine binding site of $GABA_A$ receptor. The resulting interaction models for Isovitexin and 6-C-glycoside-Diosmetin and $GABA_A$ receptor agree remarkably well with the model of Diazepam described by Bergmann et al. (2013) and Richter et al. (2012). Our data indicated strong interactions in the BZ-binding site for Isovitexin and 6-C-glycoside-Diosmetin; however, this was not the case for Vicenin-2 and Vitexin. Thus, Isovitexin and 6-C-glycoside-Diosmetin can act similarly to Diazepam at the BZ-site on $GABA_A$ receptors but without the side effect of memory-impairment (amnestic effects).

In brief, the obtained binding mode of Isovitexin orients the carbonyl oxygen at C_4, ring C, towards α_1Thr206 and α_1Ser204 in the BZ-site binding pocket. The hydroxyl oxygen at C_5-ring A is positioned in towards α_1Thr206 and α_1Ser204 in the BZ-site binding pocket. It forms a quadrilateral geometry in the α_1 subunit. The hydroxyl oxygen at C_7 of ring A is positioned in the γ_2Thr142 in the BZ-site binding pocket in a manner similar to the BZ binding mode. Ring B was positioned in a hydrophobic cavity between γ_2Phe77 and α_1Phe99. Finally, the glycoside on C_6 of ring A orients the hydroxyl groups of the sugar towards γ_2Asp56 and γ_2Glu189 (Fig. 5D). In the case of 6-C-glycoside-Diosmetin, ring B is positioned in a hydrophobic cavity between γ_2Phe77, α_1Phe99 and α_1Tyr159. The glycoside on C_6 and the hydroxyl group on C_7 of ring A orient the hydroxyl groups towards γ_2Asp56 (Fig. 5G). In addition, the anxiolytic-like effects observed for Isovitexin and 6-C-glycoside-Diosmetin are explained by strong interactions in residues α_1Thr206, α_1Ser204 and γ_2Thr142 in the BZ-binding site on $GABA_A$ receptors. It is for this reason that these flavones may act as agonists of $GABA_A$ receptors, such as Diazepam in the binding mode of Diazepam described by Bergmann and colleagues (Bergmann et al., 2013).

Similarly, Isovitexin and 6-C-glycoside-Diosmetin showed a lack of

interaction with α_1His101, which may explain the memory-enhancing effect identified in the behavioural test (Fig. 3D). Regarding this subject, in the BZ-binding mode of Diazepam, the chlorine atom of DZ interacts with α_1His101 (Fig. 5A). Thus, the occurrence of the α_1His101 interaction could justify the amnestic effect (memory-impairing) observed in Diazepam similar to the effect previously established in mutational studies in the literature (Rudolph et al., 1999; Rudolph and Mohler, 2004). In this context, this study provided novel evidence regarding memory enhancers. These observations will require confirmation using different types and mutant forms of α subunit-containing $GABA_A$ receptors.

Vicenin-2 (**2**) and Vitexin (**3**) exhibited a low probability of interaction with the $GABA_A$ BZ binding pocket (Supplementary Figs. 2A and 2D), which may be explained by the presence of glycoside at C_8 of ring A (discussions at Supplementary). Following the positioning of glycoside at C8 of ring A, a steric hindrance was observed in the binding pocket, which was in accordance with the data of a quantitative structure-activity relationships (QSAR) study with flavones on $GABA_A$ receptors (Dekermendjian et al., 1999; Marder et al., 2001).

5. Conclusion

Altogether, our findings demonstrate a concomitant anxiolytic-like and cognitive-enhancement effect produced by Isovitexin and 6-C-glycoside-Diosmetin. These flavones modulate $GABA_A$ receptors via their benzodiazepine-binding site, such as Diazepam, which may result in anxiolytic-like behaviour. We suggest that cognitive-enhancements as observed in the group treated with Isovitexin and 6-C-glycoside-Diosmetin, may be the result of a lack of interaction in α_1His101 in the BZ-binding site on $GABA_A$ receptors. Additional approaches are required to understand the mechanisms involved in these processes. These findings suggest that Isovitexin and 6-C-glycoside-Diosmetin provide novel therapeutic approaches that may be employed for anxiety disorders.

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Conflict of interest

The authors declare that the research was conducted in the absence of commercial or financial relationships that may be construed as a potential conflict of interest.

Authorship contributions

DRO conceived the study and was involved in experimental analysis and molecular modelling studies. AHT performed the molecular modelling studies. SMC, DGGR, AJC, JMC and GMR supervised the experiments, analysed the results and reviewed the manuscript. All authors have read and approved the manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2018.05.004.

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